Development of a Point-of-Care Immunochromatographic Test Based on Rhoptry Protein 14 for Serological Detection of Toxoplasma Gondii Infection in Swine

Yimin Yang

Zhejiang University

Yechuan Huang

Zhejiang University

XianFeng Zhao

Shenzhen Entry-Exit Inspection and Quarantine Bureau

Mi Lin

Zhejiang University

Lulu Chen

Zhejiang University

Mingxiu Zhao

Zhejiang University

Xueqiu Chen

Zhejiang University

Yi Yang

Zhejiang University

Guangxu Ma

Zhejiang University

Chaoqun Yao

Ross University School of Veterinary Medicine

Siyang Huang

Yangzhou University

Aifang Du (✉ afdu@zju.edu.cn)

Zhejiang University <https://orcid.org/0000-0002-3796-6621>

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Abstract

Background: *Toxoplasma gondii*, a worldwide distributed apicomplexan protozoan, can infect almost all warm-blooded animals and may cause toxoplasmosis. Globally, the prevalence of *T. gondii* in pigs varies between 10% and 60%. Therefore, the detection and prevention of swine toxoplasmosis is essential for agriculture and public health. In order to provide a point-of-care detection method for *T. gondii* infection in swine, an immunochromatographic test (ICT) was established.

Methods: A sequence corresponding to amino acids 619-1061 of *T. gondii* rhoptry protein 14 (TgROP14) was cloned from *T. gondii* cDNA and expressed in *Escherichia coli* BL21 (DE3) strain. Monoclonal antibodies (mAb) against TgROP14 were prepared by immunizing BalB/c mice with the recombinant TgROP14 (rTgROP14), and the specificity, subtype, titer and concentration of the mAb were analysed. An immunochromatographic strip (ICT) based on purified rTgROP14, recombinant protein A and mAb against TgROP14 was developed. The specificity, sensitivity, and stability of this new ICT were evaluated. Finally, 436 porcine sera sampled from Zhejiang province in China were tested using the new ICT and a commercial IHA kit to assess the relative sensitivity and specificity.

Results: rTgROP14 could be specifically recognized by positive serum of *T. gondii* but not negative serum. The mAb TgROP14-5D5 shows higher specific recognition of *T. gondii* antigens and was therefore selected for subsequent colloidal gold strip construction. The new ICT based on TgROP14 exhibited good diagnostic performance with high specificity (89.6%) and sensitivity (100%). Among 436 field porcine sera, ICT and IHA each detected 134 (30.7%) and 99 (87.5%) positive samples, respectively. The relative agreement was 87.5%.

Conclusions: This new ICT based on TgROP14 is a candidate for routine testing of *T. gondii* in the field.

Background

Toxoplasma gondii is a worldwide distributed apicomplexan, which can parasitize nearly all types of warm-blooded animals including humans [1]. Approximately one third of human population globally has been infected by *T. gondii* [2]. *T. gondii* infection in healthy adults is usually asymptomatic; however, devastating consequences often occur in the congenital infections and the people of compromised immunity [3]. The majority of human *T. gondii* infections are caused by oral ingestion of raw or inadequately cooked meat containing viable tissue cysts, or by consuming food or water contaminated with cat-shed oocysts [4, 5]. Pigs (*Sus scrofa*), as an intermediate host of *T. gondii*, have important economic and public health significance [6], and tissue cysts can survive for more than two years when most pigs are sacrificed as pork within a year [5]. Globally, the infection rate of *T. gondii* in pigs varies widely, typically between 10% and 60% [7]. China has one of the largest pork industries worldwide with the swine prevalence of *T. gondii* infection ranging from 58.1% in southern [8] to 24.5% in central [9]. Therefore, detection and prevention of swine toxoplasmosis is of great significance for agriculture and public health in China as well as the rest of the world.

Clinical manifestations of toxoplasmosis are not always obvious and specific, diagnosis of *T. gondii* infection generally relies on laboratory testing of parasites, antibodies, and/or DNA [10] using pathogenic, immunological, imaging techniques and molecular detection [11]. Immunological diagnostics have been the preferred and the most commonly used [12]. They include indirect hemagglutination test (IHA), latex agglutination test (LAT) and enzyme-linked immunosorbent assays (ELISA) [13]. These methods are commonly used even though having certain limitations such as skillful operator, expensive equipment and time consuming. The colloidal gold immunochromatographic test (ICT) has been considered suitable for rapid detection outdoors, as it is simple to operate and no need for equipment plus small in size and convenient to store [14]. Several ICTs have been developed for detection of *T. gondii* infections, including based on surface antigen 2 (SAG2) [15], surface antigen 3 (SAG3) [16] or dense granule antigen protein 7 (GRA7) [17, 18].

Rhoptry proteins of *T. gondii* (TgROPs) are potential diagnostic antigens [19, 20, 21, 22]. Among them, TgROP14 locates to the parasitophorous vacuole membrane (PVM), possibly serving as a membrane transporter in participating in the exchange of substances between the parasitophorous vacuole (PV) and the host cell cytoplasm [23]. We aimed to use TgROP14 as a key molecule in a reliable, yet quick and user-friendly diagnostic method for detection of *T. gondii* infection that can be used at point-of-care.

Methods

Parasites and cell culture

African green monkey kidney epithelial (Vero) cells (ATCC, CCL-81™), were maintained in Dulbecco's modified Eagle's medium (DMEM, Bioind) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco). Tachyzoites of RHΔ*ku80*Δ*hxcprt* strain were cultured in vitro by serial passage on Veros at 37°C and 5% CO₂, with DMEM supplemented with 2% (v/v) of FBS.

Soluble protein preparations and western blot

Freshly harvested *T. gondii* tachyzoites, were washed thrice in phosphate buffered saline (PBS), sonicated 5 × 20 s at 5 kHz, and centrifuged at 12,000 g for 10 min to collect the supernatant containing *T. gondii* soluble antigens. About 10 µg soluble protein was loaded into each of lanes and transferred to 0.22 µm PVDF membranes (Millipore, Germany). Membranes were blocked with 5% (w/v) skim milk for 1 hour at room temperature and probed with appropriate primary antibodies, followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Fudebio, China) for 1 hour. Blots were exposed using ECL substrates (Fudebio, China) and visualized using the ChemiDoc™ chemiluminescence system (Bio-Rad, USA).

Preparation of the recombinant protein TgROP14 (rTgROP14-His)

Following the manufacturer's instructions (Invitrogen, USA), total RNA of *T. gondii* RH Δ ku80 Δ hxgprt tachyzoites was extracted with TRIzol and was reverse-transcribed into cDNA using the reverse transcription kit ReverTra Ace- α -® (Toyobo, Japan). An insert corresponding to amino acids 619-1061 of TgROP14 (GenBank accession number DQ096565.1) was amplified by PCR using following primers: 5'-CCGGAATTCATGCCAGACCAGGTTATGGATTCAG-3' and 5'-CCCAAGCTTCAGCGCTTGCTTCTTCCTAGTC-3', including an *Eco*R I and a *Hind* III restriction enzyme site underlined. *Eco*R I- and *Hind* III-digested PCR products were cloned into the *Escherichia coli* expression vector pET-30a (Novagen, China). The right construct confirmed by sequencing was transformed into *E. coli* BL21 (DE3) (TaKaRa, Japan) to express rTgROP14-His by 1 mM IPTG induction at 37°C. After bacterial sonication in PBS (0.1 M, pH 7.4), rTgROP14-His was purified by Ni-NTA agarose (GE, USA). The quality of rTgROP14-His was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot (WB).

Production of mouse monoclonal antibodies against TgROP14

For mAb preparation, six-week-old female BalB/c mice (Hangzhou, Zhejiang, China) were immunized with purified rTgROP14-His and then the immunized spleen cells of mice were isolated and fused with SP2/0 myeloma cells [24]. Briefly, for the first immunization, each mouse received 100 μ g of purified rTgROP14-His mix with an equal volume of the complete Freund's adjuvant. For the second and third immunization, the mice were immunized with the same dosage of purified rTgROP14-His mixed in a 1:1 ratio with the incomplete Freund's adjuvant, both at 2 weeks interval. One week after the third immunization, a booster intraperitoneal injection was given with only 50 μ g rTgROP14-His. Three days afterwards, harvested spleen cells were fused with SP2/0 myeloma cells by polyethylene glycol.

Hybridomas were screened by ELISA for producing monoclonal antibodies (mAbs) against TgROP14 using rTgROP14-His to coat wells. ELISA-positive hybridomas were further screened by IFA. Briefly, *T. gondii* tachyzoites-infected Veros were fixed in 4% paraformaldehyde (PFA) for 15 min at 4°C, permeabilized with PBS containing 0.25% Triton X-100 for 15 min at 37°C, and then blocked in 1% bovine serum albumin (BSA) for 1 h at 37°C. Subsequently, they were incubated both for 1 h in mAbs anti-TgROP14 (1:1000) and Alexa Fluor 488-conjugated secondary antibodies (1:1000) and stained with DAPI (4',6-diamidino-2-phenylindole). Finally, the fluorescence images were obtained by a laser scanning confocal microscope (Zeiss LSM 780, Jena, Germany). Positive hybridomas identified were subcloned three times by limiting dilution method. Ascites was generated in the paraffin-primed BalB/C mice and purified using saturated ammonium sulfate [(NH₄)₂SO₄]. The quality and specificity of the mAbs were tested by SDS-PAGE and western blot.

Identification of types and epitopes' specificities of the mAbs

A mouse monoclonal antibody isotyping kit (Biodragon, China) was selected to determine the isotype of the mAbs. The epitopes' specificities of the two mAbs prepared were tested by an ELISA overlap

experiment, and additivity indexes (A.I) of different mAbs combined were calculated using methods as described before [25].

Preparation of immunoassay materials

First, colloidal gold solution was prepared. Briefly, 2.5 mL of 1% trisodium citrate solution was supplemented into boiled 100 mL of 0.01% HAuCl₄ solution by stirring thoroughly and continuously. Kept the mixture boiling for an additional 10 min after its color turned from blue to dark red. Afterwards, constantly stirred the mixture for another 5 min before the preparation completed. The prepared gold colloids were stored in dark at 4°C with 0.01% (m/v) sodium azide (NaN₃). Transmission electron microscopy was used to identify the gold colloids.

The desalted recombinant protein rTgROP14-His was used as an antibody detector after being conjugated with the colloidal gold, while staphylococcal protein A (Sangon Biotech, Shanghai) was used as the capture protein. The optimum conditions were determined as follows: 0.2 mL of purified and desalted rTgROP14-His (1.5 mg/mL) was supplemented into 20 mL of colloidal gold solution (pH 8.2). The mixture was stirred carefully for 15 min, and blocked by 10% BSA (m/v) for 1 hour. After centrifugation (12,000g, 30min), the colloidal gold–antigen conjugate from the sediment was resuspended in 2 mL dilution buffer [0.2 M tris solution (pH 8.0) with 10% BSA, 20% Sucrose, 5% trehalose and 0.2% NaN₃] and stored at 4°C. The recombinant protein A (4 mg/mL) and TgROP14-5D5 mAb (2 mg/mL) were transferred to the nitrocellulose (NC) membrane (Millipore, China) with a rate of 1 µL/cm, respectively forming the test and control lines. The strips were incubated and dried at 37°C for 1 h.

Preparation of the immunochromatographic strip

The ICT strip was assembled as showed in Fig. 1. It consisted of a sample pad, which saturated with 0.01 M PBS (pH 8.2) containing 0.1% Tween-20, a conjugate pad which was added with colloidal gold probe, an immobilized NC membrane, and an absorbent pad. Both sample pad and the conjugate pad along with the NC membrane prepared above were dried at 37°C. Pure cellulose fiber was served as the absorbent pad and the PVC plate was set as the assay membrane at the bottom of the test strip. These strips were sequentially overlapped with the sample pad, conjugate pad, fixed NC membrane and absorbent pad, cut into 4 mm width and stored with desiccators at 4°C until use.

A sample in 80 µL was added in drop to the sample pad. It passed through the NC membrane and a result showed up in 10 min. Both the test and control zones showing red lines indicated the positive result, while only the control zone appearing red denoted negative. A single red line at the test zone or no red line at all in the strip was determined as an invalid test.

Sensitivity, specificity, and stability of the immunochromatographic test

To identify the detection limit of ICT, the standard *T. gondii*-positive porcine serum were diluted in a ratio of 1:2, 1:4, 1:8 with 0.9% NaCl (pH 7.2), and the standard negative porcine serum as the negative control. The specificity of ICT was evaluated using positive porcine sera for the porcine reproductive and respiratory syndrome (American type), the swine type O foot-and-mouth disease, the swine type A foot-and-mouth disease and the swine fever conserved in our laboratory. The strips stored at 4°C for 12 weeks were used to examine the stability of this ICT.

Detection of *T. gondii* infection in field samples

436 porcine sera sampled from Zhejiang province in China were tested using the new ICT. They were also detected by the IHA kit (Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, Gansu Province, China) serving as a “reference standard” to assess the relative sensitivity and specificity of the newly developed ICT.

Results

rTgROP14-His proteins are recognized by antibodies to *T. gondii*

The recombinant protein rTgROP14-His was generated by transfecting *E. coli* BL21 (DE3) with the plasmid pET30a-TgROP14-His. It was approximately 70 kDa as confirmed by SDS-PAGE (Fig. 2a) and Western blot with anti-His antibody (Fig. 2b). Further, rTgROP14-His was recognized by mouse polyclonal antibodies to *T. gondii* (Fig. 2c), which indicated that rTgROP14-His has good immunogenicity.

TgROP14-5D5 is the candidate for colloidal gold strip

Two mAbs 1E9 and 5D5 against rTgROP14-His were characterized using the natural antigens of *T. gondii* tachyzoites by Western blot. Both detected two proteins at approximately 140 and 70 kDa (Fig. 3a), and it is possible that the upper band is the dimer of the lower band, alternatively, they were the same proteins with different post-translational modifications. They were further determined to be IgG3 and IgG2a, respectively (Table 1). The two mAbs were successfully purified by saturated ammonium sulfate as shown by the heavy chain (~55 kDa) and the light chain (~25 kDa) in SDS-PAGE (Fig. 3b). Furthermore, the value of A.I was 22.8%, which demonstrated that these two mAbs targeted the same epitope (Table 1). Specificities of these two mAbs were further analysed by IFA, and TgROP14-5D5 shows higher specific recognition of *T. gondii* antigens than TgROP14-1E9 (Fig. 3c). TgROP14-5D5 had a titer of about $1:3.3 \times 10^7$, and was selected for subsequent colloidal gold strip construction because of its higher specificity.

Table 1
Immunological characteristics of TgROP14-5D5
mAb and TgROP14-1E9 mAb.

mAb	Isotype	Titer	Additivity index
5D5	IgG2a	1:3.3×10 ⁷	22.80%
1E9	IgG3	-	

Immunochromatographic test using TgROP14-5D5 is sensitive and specific

The sensitivity of the ICT was gauged by a series of 1:2 diluted *T. gondii* positive porcine serum samples up to 1:16. The red color at the testing line can be clearly observed when the sera were undiluted to 1:4 (Fig. 4a), indicating that the detection limit of the ICT was 1:4. The specificity of the ICT was verified with porcine serum samples positive for 4 different pathogens which were often clinically observed. They included porcine reproductive and respiratory syndrome virus, swine type O and A foot-and-mouth disease virus and swine fever virus. Two red lines were only observed with the *T. gondii* positive porcine serum at the test and control zones. In contrast only the control line appeared when each of other sera was tested (Fig. 4b). Furthermore, the red line could be clearly seen in the test band exposed to positive porcine serum with the same batch of ICT strips after stored at 4°C for 12 weeks (data not shown), indicating that the test strips stored at 4°C were stable for at least 12 weeks.

Newly developed immunochromatographic test is suitable for field samples

We next compared the newly developed ICT test with a commercially available IHA as a “gold standard”. We used 436 porcine sera collected from farm pigs in Zhejiang, China. The new ICT found 134 positive sera, which was 30.7%. In contrast, IHA detected 99 positive sera, representing 22.7% of all samples (Table 2). The relative sensitivity and specificity of this newly prepared ICT were 100% and 89.6%, respectively, and the relative agreement between ICT and IHA was 87.5%.

Table 2
Sensitivity and specificity of the newly developed immunochromatographic test (ICT) using a commercially available IHA as a “reference standard”.

Test	No. (%) of positive samples	No. (%) of negative samples	Sensitivity (95% CI)	Specificity (95% CI)
ICT	134 (38.7)	302 (87.3)	100 (96.3 to 100)	89.6 (85.9 to 92.4)
IHA	99 (22.7)	337 (77.3)		

Discussion

Toxoplasma lysate antigens (TLAs), prepared from mice and/or tissue culture derived tachyzoites, were used to develop early commercial serological detection kits for diagnosis of *T. gondii* [26]. However, the methods used to produce these antigens vary significantly between laboratories or even from batch to batch in the same lab. It is important to point out that TLAs prepared from tachyzoites also contain a variety of nonparasitic substances derived from host cells. Furthermore, low productivity and potential health biohazard of native antigens cannot be ignored. Therefore, the native antigens have been gradually replaced by recombinant antigens, which eliminate the risk of laboratory infection caused by working with live tachyzoites. Advantages of using recombinant proteins include easier to be standardized, finer repeatability, and consistency of protein preparation. Over the past 30 years, many recombinant antigens of *T. gondii* as diagnostic targets have been assessed to detect specific antibodies in human serum [10, 27]. Some researchers also evaluated the feasibilities of utilizing recombinant *T. gondii* antigens for the detection of specific antibodies in animal sera [28, 29]. To date, all recombinant antigens tested in animals were obtained by the *E. coli* prokaryotic expression of *T. gondii* proteins such as SAGs, microneme proteins (MICs), rhoptry neck proteins (RONs) and ROPs, and GRAs [30].

ROPs of *T. gondii* such as ROP1 and ROP2 have been used as detection antigens for diagnosis [19, 20, 21, 22]. The recently discovered ROP14 may be a membrane transporter and likely to be located to the PVM, and participate in substance exchange between *T. gondii* and host cell [23]. In this study, the DNA sequence encoding TgROP14 minus transmembrane domain was PCR amplified and cloned, the resultant recombinant protein rTgROP14-His was obtained by the *E. coli* prokaryotic expression system. Further analyses firmly established that the rTgROP14-His was a potential candidate to be a diagnostic antigen.

Several serologic kits are commercially available for the diagnosis of *T. gondii* infection, including LAT, modified agglutination test (MAT), Western blotting test, and immunofluorescence antibody test (IFAT). All use native antigens. At present, most serologic methods require technical training and are laborious, and are hard to be used at the point-of care. ICT is not only highly sensitive and specific, but also rapid, cost-efficient and of very minimum training requirement of operators, making it applicable and attractive for field application [31]. Here, we developed an ICT using purified rTgROP14, recombinant protein A and mAb TgROP14-5D5 for serological detection of *T. gondii* in swine populations.

Several ICTs have been developed for detecting *T. gondii* infections so far. An ICT based on recombinant TgSAG2 was established for detection of anti-*T. gondii* antibodies in cat with relative sensitivity and specificity of TgICT of 100 and 94.5% compared with LAT and 97.2 and 95.8% compared with ELISA [15]. An additional ICT based on GRA7 was developed for detecting *T. gondii* infection in swine populations. The relative sensitivity and specificity were 80% and 100% when iELISA was regarded as a reference [17]. Previously, the sensitivity and specificity of a few other approaches were computed respectively: 45.9 and 96.9% for LAT, 82.9 and 90.29% for MAT, 29.4 and 98.3% for IHAT, and 72.9 and 85.9% for ELISA[32]. Compare with the above methods, the ICT developed in this study exhibited higher sensitivity but lower specificity. Current rule is that the authenticity of positiveness for *T. gondii* requires to be determined by at least two different commercial tests (e.g., MAT and ELISA), and the results acquired from a new

diagnostic method such as using a recombinant antigen are compared with those of commercial tests. By this rule, the novel ICT based on rTgROP14 and TgROP14-5D5 mAb is promising to become a practical serological diagnostic test for the clinical investigation of *T. gondii* infection at the point-of-care.

Conclusions

In this study, we showed that the rhoptry protein TgROP14 can recognize positive serum of *T. gondii* but not negative serum. The mAb TgROP14-5D5 can specifically recognize *T. gondii* antigens. The ICT using purified rTgROP14, recombinant protein A and mAb TgROP14-5D5 had good specificity and sensitivity. The novel ICT has potential for serological detection of *T. gondii* at the point-of-care in swine populations.

Abbreviations

ICT
Immunochromatography test
TgROP14
T. gondii rhoptry protein 14
mAb
Monoclonal antibodies
rTgROP14
The recombinant TgROP14
IHA
Indirect hemagglutination test
LAT
Latex agglutination test
ELISA
Enzyme-linked immunosorbent assay
SAG2
Surface antigen 2
SAG3
Surface antigen 3
GRA7
Dense granule antigen protein 7
TgROPs
Rhoptry proteins of *T. gondii*
PVM
Parasitophorous vacuole membrane
PV
Parasitophorous vacuole
DMEM

Dulbecco's modified Eagle's medium
FBS
Fetal bovine serum
PBS
phosphate buffered saline
PVDF
Polyvinylidene fluoride
HRP
Horseradish peroxidase
SDS-PAGE
sodium dodecyl sulfate-polyacrylamide gel electrophoresis
ECL
Efficient chemiluminescence
IPTG
isopropyl beta-D-thiogalactopyranoside
TLAs
Toxoplasma lysate antigens
ROP1
Rhoptry protein 1
ROP2
Rhoptry protein 2
ROPs
Rhoptry proteins
RONs
Rhoptry neck proteins
MICs
Microneme proteins
GRAs
Dense granule antigens
MAT
Modified agglutination test
IFAT
immunofluorescence antibody test.

Declarations

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Availability of data and materials

All data supporting the conclusions of this article are included within the article. Data and materials can be available upon reasonable request to the corresponding author.

Authors' contributions

AFD and YMY conceived this project and designed the experiment. YMY, YCH, ML and MXZ performed the experiment. YMY, YCH, XFZ, LLC, CQY and SYH did the data analysis. XQC and YY purchased experimental materials. YMY wrote the manuscript. CQY and GXM participated in reviewing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Animal use approval was received from Zhejiang University Experimental Animal Ethics Committee (Permit Number: ZJU201308-1-10-072). Animal researches were carried out in line with the recommendations presented in Regulations for the Administration of Affairs Concerning Experimental Animals of People's Republic of China.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹Institute of Preventive Veterinary Medicine, Zhejiang Provincial Key Laboratory of Preventive Veterinary Medicine, College of Animal Sciences, Zhejiang University, Hangzhou 310058, PR China. ²Animals & Plant Inspection and Quarantine Technology Center of Shenzhen Customs, Shenzhen, Guangdong 518045, China. ³Ross University School of Veterinary Medicine and One Health Center for Zoonoses and Tropical Veterinary Medicine, Ross University School of Veterinary Medicine, P.O. Box 334, Basseterre, St.

Kitts, West Indies. ⁴Institute of Comparative Medicine, College of Veterinary Medicine, Yangzhou University, and Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonosis, and Jiangsu Key Laboratory of Zoonosis, Yangzhou, Jiangsu Province 225009, PR China.

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Figures

Figure 1

The component and illustration of the ICT strip.

Figure 2

Analysis of the recombinant protein rTgROP14-His by SDS-PAGE (A) and Western blot (B & C). M: Protein marker. Lane 1: rTgROP14-His. The primary antibodies in panel B and C were anti-His antibody and polyclonal mouse antibodies to *T. gondii*, respectively.

Figure 3

Identification of TgROP14-5D5 mAb and TgROP14-1E9 mAb. (A) Western blot analysis of two mAbs reacted with *T. gondii* lysate antigens. Total soluble proteins of *T. gondii* tachyzoites (10 μ g) were loaded into each of lanes 1 & 2. The primary antibodies were mAb 1E9 and 5D5 to TgROP14, respectively. M: Protein marker. (B) SDS-PAGE analysis of two mAbs before and after purification. Lane 1 and 2: 1E9 mAb after purification and before purification, respectively; Lane 3 and 4: 5D5 mAb after purification and before purification, respectively; M: Protein marker. (C) IFA assay of two mAbs. Vero cells were used after

infection with *T. gondii* tachyzoites for 24 h. The primary antibodies and Alexa Fluor 488-conjugated secondary antibodies for labeling were used in 1:1000 dilution. Confocal micrographs were shown.

Figure 4

Sensitivity and specificity of the ICT strip. (A) Sensitivity of the developed ICT. Lane 1: Negative control; Lane 2-6: Standard *T. gondii* positive porcine serum, undiluted, 1:2, 1:4, 1:8, and 1:16 dilution, respectively.

(B) Specificity of the developed ICT using sera of pigs infected with different pathogens as specified. Lane 1: Negative control; Lane 2: *Toxoplasma gondii*; Lane 3: Porcine reproductive and respiratory syndrome virus; Lane 4: Swine type O Foot-and-mouth disease virus; Lane 5: Swine type A Foot-and-mouth disease virus; Lane 6: Swine fever virus.

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